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Genomic Structure of Lowland and Highland Ecotypes of *Arabidopsis halleri* subsp. *gemmifera* (Brassicaceae) on Mt. Ibuki

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Arabidopsis thaliana (Brassicaceae) and its closely related species serve as model systems for evolutionary studies. Although much information about the genome of the species of *Arabidopsis* has been collected, knowing the genomic structure of targeted systems is required for determining which genes are involved in adaptation to particular environments. In this study using amplified fragment length polymorphisms (AFLP), we determined the genomic structure of *A. halleri* subsp. *gemmifera* (Brassicaceae) on Mt. Ibuki, Japan, where two ecotypes adapted to lowland and highland environments grow. Based on 273 loci (4.4% of the loci were outliers), genotypes of 17 and 14 individuals of lowland and highland ecotypes, respectively, were determined. When excluding outliers, genetic differentiation between the two ecotypes was low (F_{ST} = 0.017). The genotypes of each ecotype, however, were distinguishable using principle coordinate analysis. The genome-wide investigation of the two ecotypes of *A. halleri* subsp. *gemmifera* on Mt. Ibuki therefore suggests that morphological divergence as well as adaptation to local environments between lowland and highland ecotypes occurred recently and the two ecotypes share a similar genomic structure. These two ecotypes are therefore appropriate for studies to reveal the genetic basis of morphological divergence as well as local adaptation using genomic information from *A. thaliana*.

Keywords: AFLP, *Arabidopsis halleri* subsp. *gemmifera*, ecotype, genomic structure, Mt. Ibuki

Understanding the genetic basis of evolution is one of the major topics in evolutionary biology. For this purpose, finding genes involved in adaptation to local environments and/or adaptive traits is a first important step. It is difficult, however, to conduct such genetic studies on most wild species due to the lack of knowledge about their genome. *Arabidopsis thaliana*, which is a model plant used in developmental studies, is one of the candidates for unraveling the genetic basis of adaptation to natural variation (Mitchell-Olds & Schmitt 2006). Although the genetic basis of several adaptive traits has been investigated in *A. thaliana* (e.g., Bergelson *et al.* 2001, Le Corre 2005), a more generalized understanding is needed to better comprehend the genetic basis of adaptation (Ehrenreich & Purugganan 2006). Recently, other species of *Arabidopsis* (*A. lyrata, A. halleri*) have also been used as candidates for determining the genetic basis of natural variation (e.g., Hanikenne *et al.* 2008, Kuittinen *et al.* 2008), since these species have interesting ecological traits (Mitchell-Olds 2001). An ecologically interesting system in *Arabidopsis* would be a good model for studying the genetic basis of adaptation.

Arabidopsis halleri subsp. *gemmifera* (Brassicaceae) is a self-incompatible, clonal, perennial

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herb of the Russian Far East, northeastern China, Korea, Taiwan, and Japan (Al-Shehbaz and O'Kane 2002). Mt. Ibuki (1377 m), in central Japan, harbors two ecotypes that grow in lowland and highland habitats, respectively. The highland ecotype has more trichomes on the leaves and stems than the lowland ecotype. Until the work of Al-Shehbaz & O'Kane (2002), the two ecotypes were treated as *Arabis gemmifera* var. *gemmifera* and *Arabis gemmifera* var. *alpicola*. These two ecotypes therefore provide an appropriate system for determining the genetic basis of adaptation to lowland and highland environments.

To determine the genes responsible for adaptation to different environments from a reverse genetic approach, knowledge of the genetic structure throughout the entire genome is required (Luikart *et al.* 2003, Nordborg *et al.* 2006). Because little is known about the genetic structure of these two ecotypes, however, whether they originated recently and harbor similar genomic structures or are ancient and should be assigned to genetically different groups remains unclear. If the former is true, then slight genetic differentiation would be reflected in the two ecotypes. In contrast, if the latter, then strong genetic differentiation would be present throughout the genome. We therefore used amplified fragment length polymorphisms (AFLP) to determine the genetic structure throughout the entire genome of the two ecotypes of *A. halleri* subsp. *gemmifera* on Mt. Ibuki.

Materials and Methods

Sampling and DNA extraction

Leaves of lowland and highland ecotypes of *Arabidopsis halleri* subsp. *gemmifera* were collected from 17 individuals growing between ca. 350–400 m and from 14 individuals growing between 1100–1320 m. Sampling was conducted along a hiking path on Mt. Ibuki during the summer of 2007. All samples were separated by at least 4 m. The leaves were dried in silica gel and stored at room temperature. DNA was extracted using DNeasy (QIAGEN) and dissolved in 100μL of buffer.

AFLP fingerprinting

The AFLP protocol followed the procedure described by Vos *et al.* (1995) with minor modifications, i.e., we used nonradioactive fluorescent dye-labeled primers (Applied Biosystems, Foster City, CA, USA) for selective amplification on an ABI 3100 automated DNA sequencer (Applied Biosystems). We randomly selected 17 individuals to ascertain the reproducibility of the fragments. After the extracted DNA was digested with the restriction enzymes *Eco*RI and *Mse*I, it was ligated to double-stranded *Eco*RI and *Mse*I adapters (Vos *et al.* 1995). The ligated sample was pre-amplified with pre-selective primers (Applied Biosystems) and then selectively amplified using 17 pairs of primers (E-AAG/M-CAA, E-AAC/M-CAC, E-ACC/M-CAC, E-AGG/M-CAG, E-ACC/M-CAT, E-AAG/M-CAT, E-ACT/M-CTA, E-ACG/M-CTA, E-ACT/M-CTC, E-AGC/M-CTC, E-ACA/M-CTG, E-AGC/M-CTG, E-AAC/M-CTT, E-AGG/M-CTT, E-AAG/M-CTA, E-ACT/M-CAA, and E-ACA/M-CAA). For each individual, 1.5 μL of the amplified products were combined with 0.2 μL of GeneScan ROX 500 (Applied Biosystems) and 9.8 μL of Hi-Di formamide and run on an ABI 3100 capillary sequencer (Applied Biosystems) using a 50-cm capillary and POP6 polymer. The presence or absence of each fragment within each individual was scored and assembled as a binary data matrix using GeneMapper version 3.5 (Applied Biosystems).

Data analyses

The relationships among individuals were revealed by principal coordinate analysis (PCoA) based on the Jaccard distance between individuals using NTSYS-PC 2.02 (Rohlf 1998). The genetic differentiation between ecotypes (F_{ST}) was estimated and the significance was evaluated with 10,000 permutations using AFLP-SURV version 1.0 (Vekemans *et al.* 2002). Because a part of the AFLP loci would be involved in local adaptation and their genetic structure may have been influenced by natural selection, excluding non-neutral loci (outliers) was necessary to determine neutral genetic structures (Luikart *et al.*

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2003). All analyses were thereby based on all loci, loci without outliers, and outlier loci alone (see below).Whether loci evolved under neutrality was tested using the program FDIST2 (Beaumont & Nichols 1996). This method is based on the expectation that F_{ST} and its variants among the loci depend on the heterozygosity of the loci and determines outliers following the simulated distribution of F_{ST} under the null hypothesis of neutral genetic drift with migration. The neutral distribution of F_{ST} was estimated with 100,000 simulated loci, from which the 95% confidence level of F_{ST} was determined. Loci with an F_{ST} higher than the confidence level were considered to be outliers.

Results

Using 17 primer combinations, 273 reproducible fragments were detected in *Arabidopsis halleri* subsp. *gemmifera* (error rate 0.0%), which distinguished all individuals from each other. The distribution of F_{ST} showed that 12 loci (4.4%) had greater than 95% confidence and did not evolve under neutrality (Fig. 1).

The relationships of the individuals were determined using PCoA (Fig. 2). When all loci were analyzed, the genotypes of the highland and lowland ecotypes were mostly distinguishable (Fig. 2a). Weak but significant genetic differentiation between ecotypes was detected (F_{ST} = 0.072, P < 0.001). This genetic differentiation, however, was much weaker when the analyzed loci excluded the outliers (F_{ST} = 0.017, P < 0.05). In spite of the very weak genetic differentiation, two ecotypes appear to be distinguishable. In contrast, when the outlier loci were analyzed, a greater genetic differentiation was detected (F_{ST} = 0.309, P < 0.001) and the two ecotypes were more readily distinguishable, albeit with some overlap (Fig. 2c).

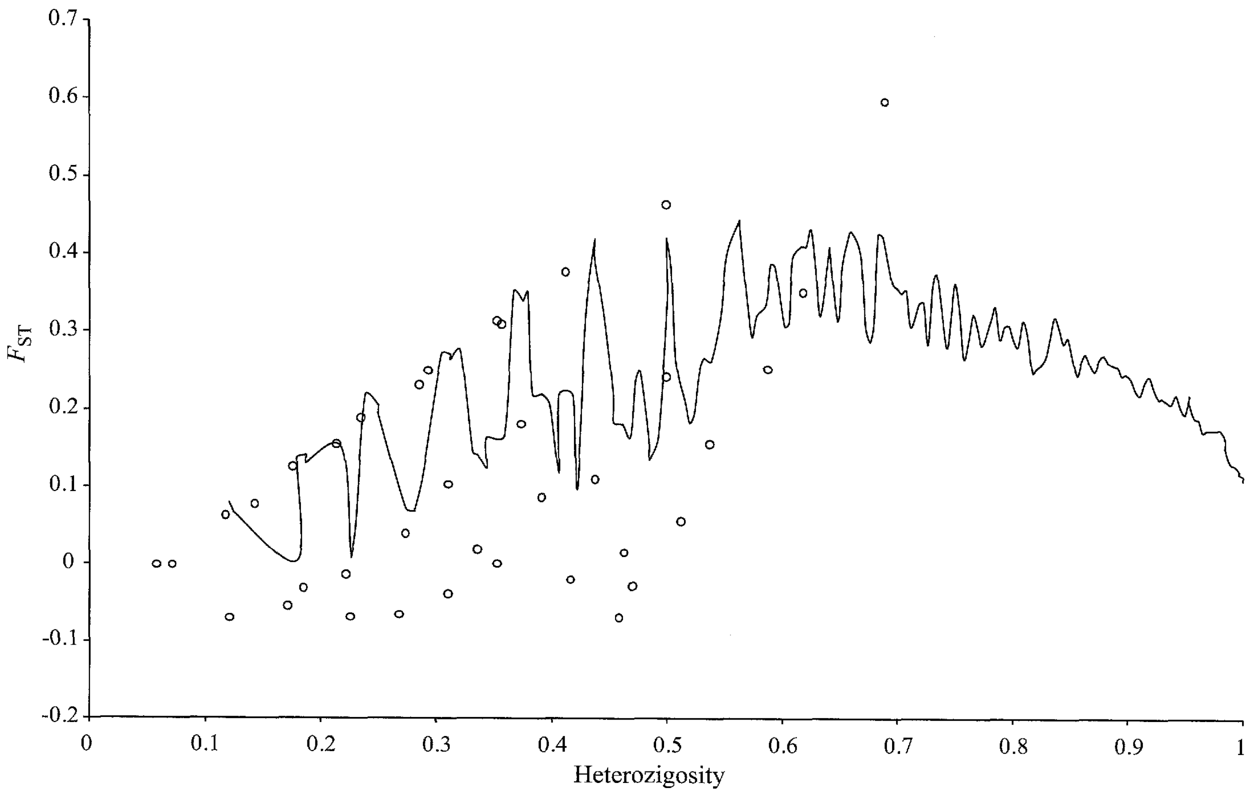

FIG. 1. Distribution of F_{ST} values against heterozygosity at each AFLP locus. Line represents 95% higher confidence interval; loci above line were determined to be outliers.

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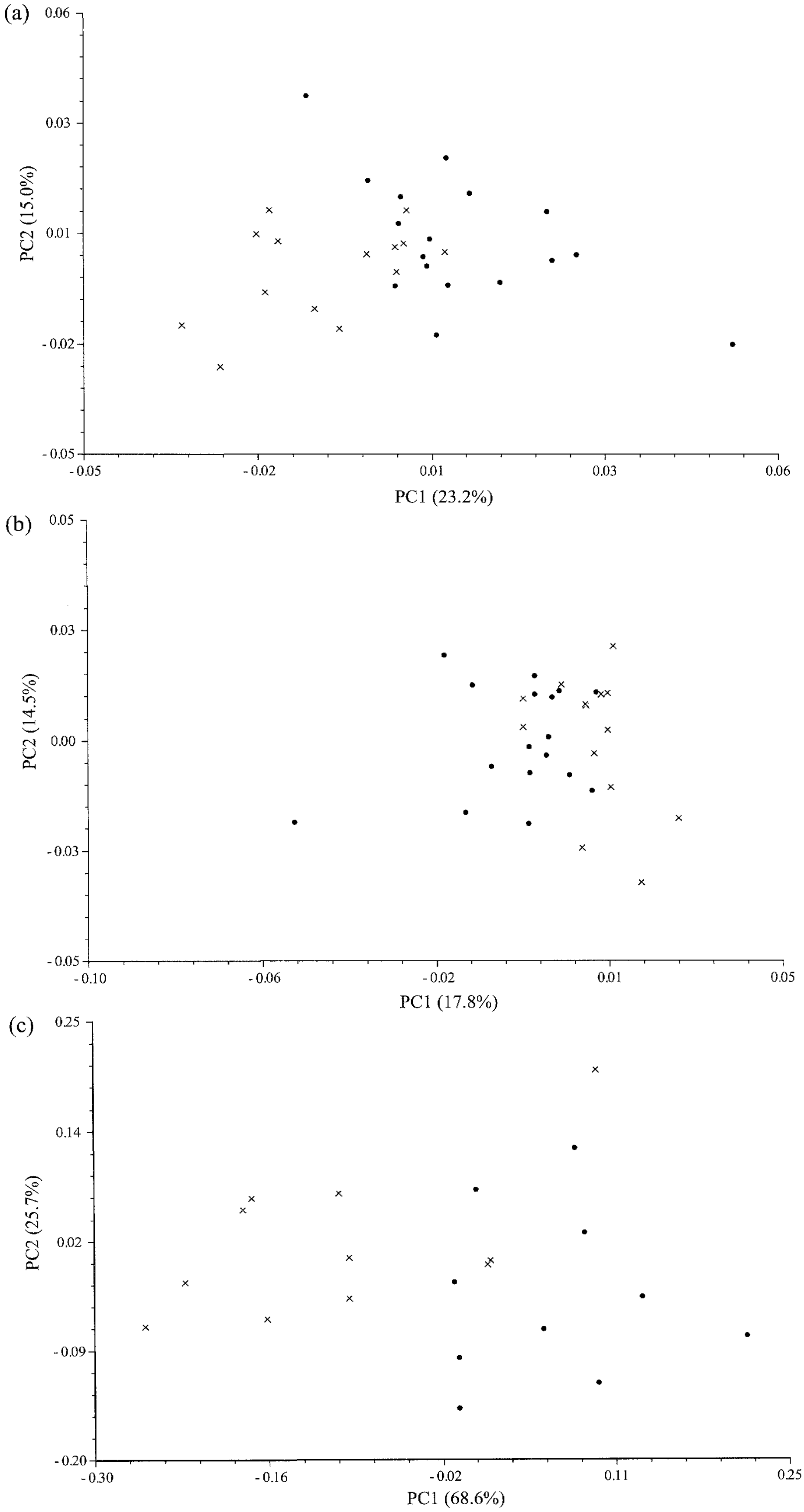

FIG. 2. Results of principle coordinate analysis (PCoA). Circles and crosses represent individuals assigned to highland and lowland ecotypes, respectively. Results are shown for (a) all loci, (b) loci excluding outliers, and (c) outlier loci alone.

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Discussion

We determined the genetic structure of *Arabidopsis halleri* subsp. *gemmifera* on Mt. Ibuki using three data sets (all loci, loci excluding outliers, outlier loci). The contrasting patterns among the loci used indicate that outliers involved in local adaptation might disturb the neutral genetic structure, resulting in an overestimate of the genetic differentiation between ecotypes. This implies that excluding outliers from multi loci data is important for elucidating the neutral genetic structure and inferring that population history was not influenced by natural selection.

After removing the outliers, two ecotypes of *Arabidopsis halleri* subsp. *gemmifera* were weakly differentiated (F_{ST} = 0.017). The present genome-wide investigation therefore revealed that the two ecotypes were not differentiated from each other across most of the genome, but harbored a similar genomic constitution. This slight genetic differentiation is inconsistent with the hypothesis that two ecotypes, ancient in origin, harbor strong genetic differentiation. Frequent gene exchanges between two genetically differentiated ecotypes, however, would also result in the absence of genetic differentiation. Where gene exchange is frequent, the genotypes of each ecotype should be highly similar. In contrast, our PCoA showed that each ecotype was genotypically distinguishable based on genome wide polymorphisms (Fig. 2b). Frequent gene exchanges between genetically differentiated ecotypes therefore makes it difficult to explain the present slight genetic differentiation between the two ecotypes. Our genome-wide investigations suggest that morphological divergence between two ecotypes may have originated recently and that only a few genes may be involved in morphological divergence as well as in the adaptation to two different ecotypes.

In the present investigation, twelve loci (4.4%) were assigned into outliers whose patterns of polymorphism did not follow neutral expectation. It is difficult to conclude that these loci as well as ca. 5.0% of the genome are involved in local adaptation between ecotypes, because the detailed function of the outliers remains unclear, and the outliers were sometimes detected by statistical error (5% type I error). Further analyses are therefore necessary to elucidate the detailed genetic basis of local adaptation between the two ecotypes. Nevertheless, our finding of similar genomic structures between the two ecotypes of *Arabidopsis halleri* subsp. *gemmifera* on Mt. Ibuki and the recent origin of morphological divergence strongly suggests that these two ecotypes are appropriate systems for studying the genetic basis of morphological divergence as well as local adaptation between lowland and highland environments using genomic information from *A. thaliana.*

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